FR167653 diminishes infarct size in a murine model of myocardial ischemia-reperfusion injury

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Objective: During myocardial ischemia-reperfusion injury, p38 mitogen-activated protein kinase is activated. We examined the effect of a highly specific inhibitor of p38 mitogen-activated protein kinase, FR167653, in an experimental model of regional myocardial ischemia-reperfusion.

Methods: CD-1 mice received FR167653 intraperitoneally 24 hours before 30 minutes of transient occlusion of the left anterior descending artery, followed by 120 minutes of reperfusion. The p38 mitogen-activated protein kinase activation and kinase activity were determined by Western blotting with monoclonal antibodies for the phosphorylated from of p38 mitogen-activated protein kinase or its substrate, activating transcription factor 2. Nuclear factor κB activity was measured by detecting translocation of nuclear factor κB to the nucleus. The expression of inflammatory cytokines was measured by ribonuclease protection assay.

Results: Pretreatment of mice with FR167653 before myocardial ischemia-reperfusion resulted in a reduction in p38 mitogen-activated protein kinase phosphorylation ($P = .018$), an inhibition of p38 mitogen-activated protein kinase activity ($P = .047$), a smaller amount of nuclear factor κB ($P = .001$), and a decrease in the expression of inflammatory cytokines (tumor necrosis factor α: $P = .023$, interleukin 1β: $P = .038$, monocyte chemotactic protein 1: $P = .0001$) in the heart and the development of a significantly smaller infarct ($P = .0069$) relative to hearts from mice treated with vehicle alone. Activation of c-Jun N-terminal kinase and extracellular signal–regulated kinase were observed after myocardial ischemia-reperfusion without inhibition by FR167653.

Conclusion: We conclude that FR167653 selectively inhibits p38 mitogen-activated protein kinase activation and activity during regional myocardial ischemia-reperfusion injury and efficaciously reduces infarct size (by 73.6%). Thus p38 mitogen-activated protein kinase inhibition may have a role in the treatment of myocardial ischemia-reperfusion.

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Myocardial ischemia-reperfusion injury is a major risk factor for patients undergoing treatment for cardiac disease, including percutaneous coronary angioplasty, coronary artery bypass grafting, heart and lung transplantation, and other cardiac procedures that require cardiopulmonary bypass.[1-4] Although the pathophysiology of myocardial ischemia-reperfusion injury remains largely unknown, activation of mitogen-activated protein kinases (MAPKs), including p38 MAPK, c-Jun N-terminal kinase (JNK), and extracellular signal–regulated kinase (ERK), are early events. Both p38 MAPK and JNK are associated with cellular responses to stress, whereas ERK activity is associated with growth and development processes in the cell. Once activated, the MAPKs phosphorylate and activate other intracellular mediators, resulting in the transcription of genes that encode such proteins as adhesion molecules, proinflammatory mediators, procoagulant molecules, and vasoactive substances.[5-10] This sequence of events may progress to irreversible tissue injury caused by an acute inflammatory response.[11-14]

Recently, FR167653* has been shown to be a specific p38 MAPK inhibitor without affecting cyclooxygenase (COX) or other MAPKs.[15] It competes with adenosine triphosphate (ATP) at the ATP-binding site of only p38 MAPK.[15]

We hypothesize that myocardial ischemia-reperfusion stress activates MAPKs, with subsequent activation of nuclear factor (NF) κB, resulting in enhanced cytokine expression associated with myocardial ischemia-reperfusion injury. Further, we postulate that specific inhibition of p38 MAPK with FR167653 will attenuate myocardial ischemia-reperfusion injury through inhibition of NF-κB. In this study, we evaluated the effect of FR167653 on myocardial ischemia-reperfusion injury in mice.

Materials and Methods

Animals and Experimental Design

Male CD-1 mice weighing 20 to 25 g (Charles River Laboratory, Inc, Wilmington, Mass) were divided into three groups: (1) the FR167653 group in which 200 μL of FR167653 (2 mg/kg dissolved in saline solution; Fujisawa Pharmaceutical Co, Ltd, Osaka, Japan) was administered intraperitoneally 24 hours before 30 minutes of ischemia followed by 120 minutes of reperfusion, (2) a vehicle group in which 200 μL of saline solution was administered intraperitoneally 24 hours before myocardial ischemia-reperfusion, and (3) a control group in which 200 μL of saline solution was administered intraperitoneally 24 hours before sham operation.

A mouse model of in situ regional myocardial ischemia-reperfusion was used as previously described elsewhere.[16] In brief, the mice were intubated and placed under mechanical ventilation after undergoing general anesthesia with pentobarbital sodium (100 mg/kg, intramuscularly). A lateral thoracotomy was performed to expose hearts, and myocardial ischemia was produced by ligating the left anterior descending coronary artery (LAD) with a 7-0 silk suture. After 30 minutes of ischemia, the occlusive snare was released to initiate reperfusion for 120 minutes. Sham-operated control mice underwent the same surgical procedures except that the suture that was passed under the LAD was not tied. Molecular analysis end points were as follows: 5 minutes of reperfusion for MAPK activation, p38 MAPK activity and IκBα phosphorylation; 60 minutes of reperfusion for NF-κB activity; and at the completion of reperfusion for messenger RNA (mRNA) to assess cytokine expression. Hearts were rapidly explanted and the left ventricle was dissected free, rinsed in 0.9% saline solution, snap-frozen in liquid nitrogen, and then stored at −80°C until subsequent analysis, whereas area at risk and infarct size were determined at the completion of reperfusion as previously described elsewhere.[16]

All animals were maintained in accordance with the "Guide for the Care and Use of Laboratory Animals" (http://www.nap.edu/catalog/5140.html) and also with the Guideline of the Animal Care and Use Committee of the University of Washington.

Western Blotting Assay for Phosphorylation of MAPKs and IκBα

Whole-cell protein, extracted from frozen tissue samples with ice-cold lysis buffer (Cell Signaling Technology, Inc, Beverly, Mass), were stored at −80°C until the time of assay. A 20-μg portion of whole-cell protein was loaded onto 15% dodecylsulfate-polyacrylamide electrophoretic gels and transferred to polyvinylidene difluoride membranes. The membranes were immunoblotted with primary antibodies in PhosphoPlus p38 MAPK (Thr180/Tyr182), JNK (Thr180/Tyr182), ERK (Thr180/Tyr182), and IκBα (Ser32) Antibody Kits (1:1000; Cell Signaling Technology). Immunoreactivity was quantitated with enhanced chemiluminescence and determined with densitometry (NIH Image 1.62; National Institutes of Health, Bethesda, Md). The ratio of phospho-MAPK to total MAPK, or phospho-IκBα, immunoreactivity was determined for each sample, and the results are expressed as fold-increase with respect to control.

p38 MAPK Activity Assay

The p38 MAPK activity was measured with the p38 MAPK Assay Kit (Cell Signaling Technology) combining immunoprecipitation, kinase assay, and immunoblotting. The results are shown as the immunoreactivity of phosphorylated activating transcription factor (ATF) 2 (Thr71), detected with enhanced chemiluminescence and determined with densitometry (NIH Image 1.62). Results are expressed as multiples of increase in activation with respect to control.

Electrophoretic Mobility Shift Assays for NF-κB Activity

Myocardial nuclear proteins from frozen tissue samples were isolated as previously described.[16] A 10-μg portion of nuclear protein was incubated in a binding reaction with a ^{32}P–end-labeled, double-stranded oligonucleotide containing the human and rodent consensus NF-κB binding sequence, 5'-AGTTGAGGGGACTTTCCCAGGC-3' (Promega Co, Madison, Wis) and subjected to

*1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8 (4-pyridyl) pyrazoro (5-1-c)(1,2,4) triazin-2-yl]–2-phenylethanedione sulfate monohydrate.

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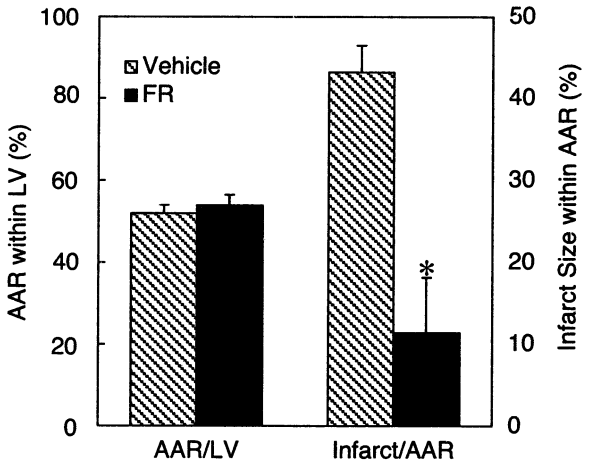

Figure 1. FR167653 blocks myocardial ischemia-reperfusion. CD-1 mice were subjected to myocardial ischemia-reperfusion injury, and area at risk *(AAR)* as a percentage of left ventricle *(LV)* and infarct size as a percentage of area at risk were determined as described in Materials and Methods section for control mice that received vehicle alone *(cross-hatched bars)* or vehicle with FR167653 (*FR,* 2 mg/kg, *black bars*). Values represent mean ± SD of 5 animals; *asterisk* indicates $P < .01$ versus vehicle.

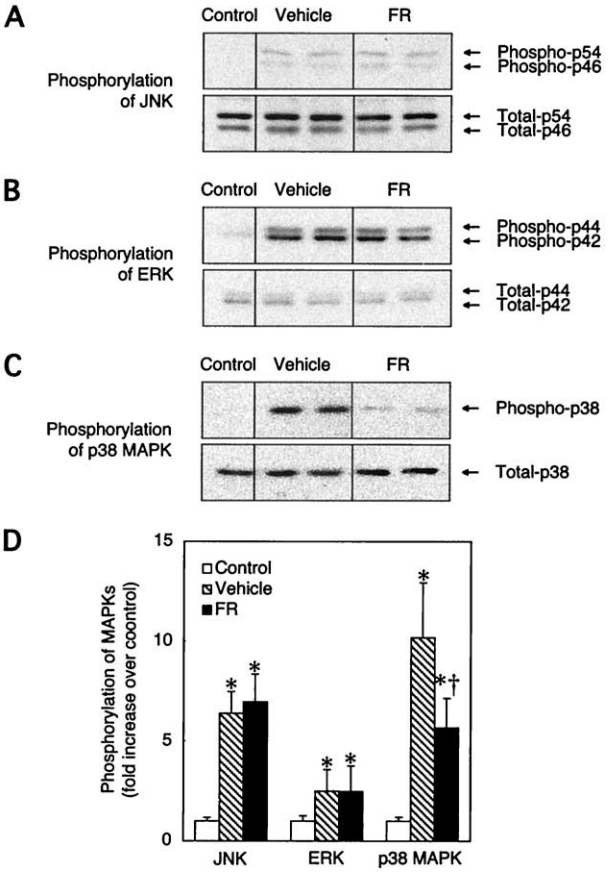

Figure 2. Effects of FR167653 *(FR)* on myocardial MAPK activation after ischemia-reperfusion. Western blotting analyses of phospho-JNK and total JNK (A), ERK (B), and p38 MAPK (C) expressions. D, Phosphorylation of MAPKs. Values represent mean ± SD of 5 animals; *asterisk* indicates $P < .001$ versus control; *dagger* indicates $P < .05$ versus vehicle.

electrophoresis in native 6% nondenaturing polyacrylamide gels. Densitometry was performed with NIH Image 1.62. Results are expressed as fold-increase in activation with respect to control.

Ribonuclease Protection Assay

Total RNA was isolated from frozen tissue samples by guanidium thiocyanate-phenol-chloroform method. RNA from each tissue was evaluated with RPA III Ribonuclease Protection Assay Kit (Ambion, Inc, Austin, Tex) and customized mouse cytokines template (Riboquant Multi-Probe Template Set; BD Biosciences Pharmingen, San Diego, Calif) according to the manufacturer's protocol. Densitometric analysis was done with NIH Image 1.62. Results are expressed as multiples of increase with respect to control after the amount of mRNA for each cytokine was normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA.

Statistical Analysis

All data are expressed as mean ± SD. The significance of the difference between group means was analyzed by analysis of variance with post hoc comparisons by Scheffé protected least-significant difference test. All statistical analyses were done with StatView 6.0 software (SAS Institute, Inc, Cary, NC).

Results

Effect of p38 MAPK Inhibition on Myocardial Infarct Size After Ischemia-Reperfusion

The ratio of area at risk to left ventricle did not differ between vehicle-treated and FR167653-treated groups. Pretreatment with FR167653 significantly reduced infarct size within the area at risk by 73.6% relative to vehicle pretreatment (FR167653 vs vehicle: 11.4% ± 6.8% vs 43.2% ± 3.3%, $P = .0069$; Figure 1).

Myocardial MAPK Activation After Ischemia-Reperfusion

Phosphorylation of JNK was significantly increased in both groups after myocardial ischemia-reperfusion compared with the control group (FR167653: 6.97 ± 1.38-fold increase, $P < .001$, vehicle: 6.39 ± 1.08-fold increase, $P < .001$); there was no significant difference between FR167653 and vehicle groups. Phosphorylation of ERK was also significantly increased in both groups after myocardial ischemia-reperfusion relative to the control group (FR167653: 2.48 ± 1.28-fold increase, $P < .001$, vehicle: 2.50 ± 1.08-fold increase, $P < .001$), and there was no difference between those that received FR167653 pretreatment versus pretreatment with vehicle. Myocardial ischemia-reperfusion significantly increased phospho-p38

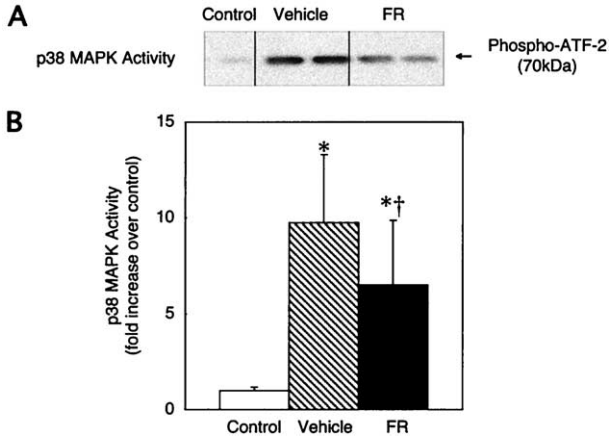

Figure 3. Effects of FR167653 *(FR)* on myocardial p38 MAPK activity after ischemia-reperfusion. A, Western blotting analysis of phospho-ATF-2 expression after immunoprecipitation and kinase assay for p38 MAPK activity. B, p38 MAPK activities. Values represent mean ± SD of 5 animals; *asterisk* indicates $P < .001$ versus control; *dagger* indicates $P < .05$ versus vehicle.

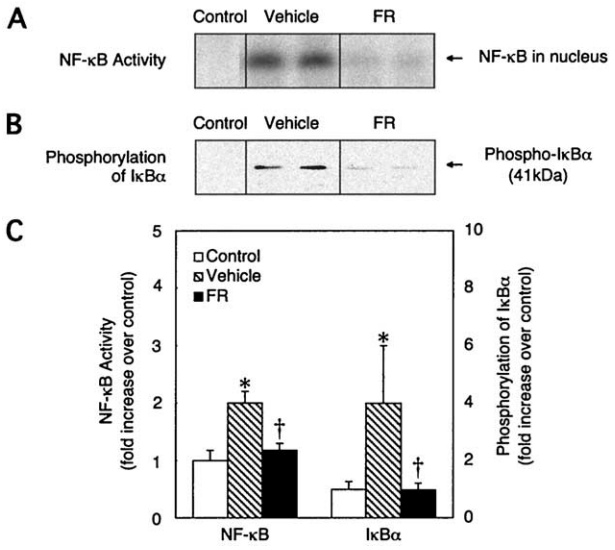

Figure 4. Effects of p38 MAPK inhibition by FR167653 *(FR)* on myocardial NF-κB activation after ischemia-reperfusion. A, Electrophoretic mobility shift assay of NF-κB activation. B, Western blotting analysis of phospho-IκBα expression as indirect indicator of NF-κB activation. C, NF-κB activities and phosphorylation of IκBα. Values represent mean ± SD of 5 animals; *asterisk* indicates $P < .001$ versus control; *dagger* indicates $P < .05$ versus vehicle.

MAPK relative to control (FR167653: 5.68 ± 1.46-fold increase, $P < .001$, vehicle: 10.17 ± 2.77-fold increase, $P < .001$). In contrast to JNK and ERK activation, FR167653 pretreatment significantly reduced the activation of p38 MAPK ($P = .018$ vs vehicle; Figure 2). Myocardial ischemia-reperfusion also significantly increased p38 MAPK activity, which was measured directly by detecting phosphorylation of a p38 MAPK substrate, ATF-2, in both groups relative to the control group (FR167653: 6.51 ± 3.36-fold increase, $P < .001$, vehicle: 9.74 ± 3.56-fold increase, $P < .001$). Pretreatment of mice with FR167653 before myocardial ischemia-reperfusion, however, significantly attenuated p38 MAPK activity relative to vehicle-treated animals ($P = .047$, Figure 3).

Effect of p38 MAPK Inhibition on Myocardial NF-κB Activation After Ischemia-Reperfusion

Relative to the control group, in vehicle-pretreated animals, there was a significant rise in NF-κB activation, as determined by assay of NF-κB translocation to the nuclear fraction (2.00 ± 0.20-fold increase, $P < .001$). Pretreatment with FR167653, however, abrogated this activation (1.18 ± 0.12-fold increase, $P = .001$ vs vehicle). Phosphorylation of IκBα, which is required for NF-κB activation, was significantly suppressed by FR167653, consistent with inhibition by FR167653 of p38 MAPK-mediated NF-κB activation (FR167653: 0.98 ± 0.23-fold increase, $P = .028$ vs vehicle, vehicle: 3.99 ± 2.01-fold increase, $P < .05$ vs control; Figure 4).

Effect of p38 MAPK Inhibition on Myocardial Inflammatory Cytokine Gene Expression After Ischemia-Reperfusion

Myocardial ischemia-reperfusion stress significantly increased mRNA levels of tumor necrosis factor (TNF) α, interleukin (IL) 1β, and monocyte chemotactic protein (MCP) 1 in vehicle-pretreated mice (TNF-α: 1.56 ± 0.27-fold increase, $P < .05$ vs control, IL-1β: 3.46 ± 1.14-fold increase, $P < .01$ vs control, MCP-1: 7.46 ± 0.48-fold increase, $P < .001$ vs control). Pretreatment with FR167653 significantly attenuated this inflammatory cytokine response (TNF-α: 0.65 ± 0.18-fold increase, $P = .023$ vs vehicle, IL-1β: 1.57 ± 0.14-fold increase, $P = .038$ vs vehicle, MCP-1: 2.84 ± 0.77-fold increase, $P = .00015$ vs vehicle; Figure 5). FR167653 had no effect on expression of lymphotactin; macrophage inflammatory protein 1α, 1β, and 2; RANTES (regulated on activation, normal T-cell expressed and secreted), T-cell activation gene 3, or eotaxin (data not shown).

Discussion

This study demonstrates that myocardial ischemia-reperfusion injury in a mouse model causes rapid phosphorylation and activation of p38 MAPK and JNK, subsequent NF-κB activation, cytokine expression in the ischemia-reperfusion-injured myocardium, and ultimately infarction. A limitation of this study is that we measured only myocardial infarction

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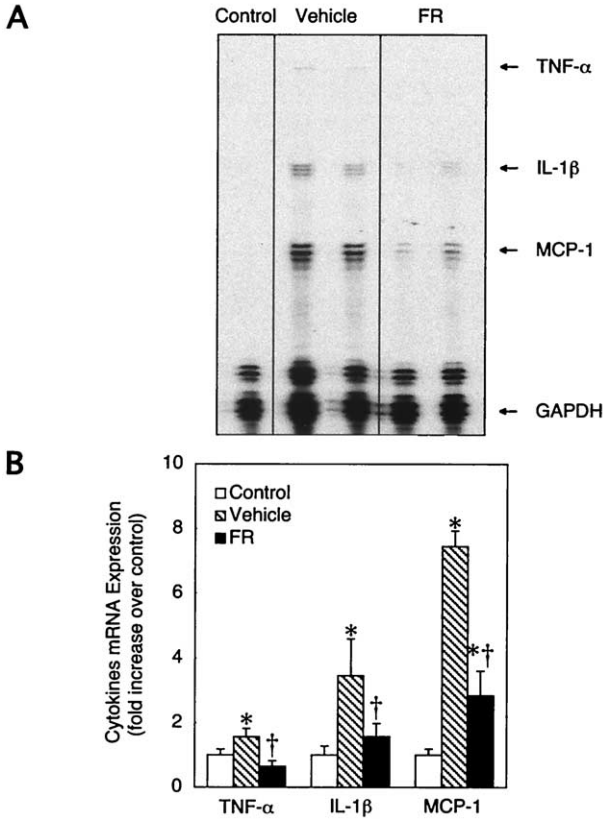

Figure 5. Effects of p38 MAPK inhibition by FR167653 *(FR)* on myocardial inflammatory cytokine gene expression after ischemia-reperfusion. A, Ribonuclease protection assay of TNF-α, IL-1β, MCP-1, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, internal control) mRNA expressions. B, Vertical axis denotes multiplicity of increase relative to control after amount of mRNA for each cytokine was normalized to that of glyceraldehyde-3-phosphate dehydrogenase mRNA. Values represent mean ± SD of 5 animals; *asterisk* indicates $P < .05$ versus control; *dagger* indicates $P < .05$ versus vehicle.

as an end point of regional ischemia-reperfusion injury. Although possible to a limited degree in mice, acquisition of myocardial functional data is exceedingly difficult and potentially unreliable. We therefore relied on infarct size as our end point measurement of regional myocardial ischemia-reperfusion injury. These cellular events, with the exception of JNK and ERK activation, were suppressed by pretreatment of mice with FR167653, which specifically blocks p38 MAPK activation by competing with ATP at the ATP-binding site of only p38 MAPK.

Previous studies have used SB203580 as a p38 MAPK inhibitor.[17-19] The actions of SB203580, however, are not confined to selective inhibition of p38 MAPK. SB203580 has been reported to inhibit thromboxane synthase, COX-1 and -2,[20] and JNK,[21] all of which may directly affect injury after myocardial ischemia-reperfusion. This nonspecific action of SB203580 may be the reason for the conflicting observations that have been reported regarding the role of p38 MAPK in myocardial ischemia-reperfusion injury.

The p38 subfamily of MAPKs consists of four isoforms (α, β, γ, and δ); only p38α and p38β have been detected in the cardiac tissue.[22] MAPK kinases (MKKs) selectively activate p38 MAPK in different cell types and exhibit isoform specificity: MKK3 activates only α and γ isoforms, whereas MKK6 can activate α, β, and γ isoforms.[22] Recent studies revealed a different role for p38α and β isoforms in apoptotic responses and cell survival: a negative role for p38α and positive role for p38β.[10,23] One possible explanation for these divergent functions of p38 MAPK isoforms is that they mediate phosphorylation of different downstream substrates that have different roles in apoptosis. Ischemia-reperfusion and ischemic preconditioning may differentially activate p38 MAPK isoforms, accounting for the controversies in previous studies. Ischemia-reperfusion has been shown to activate p38α.[5,24,25] Myocytes that ectopically expressed p38α and p38β showed activation of p38α during sustained simulated ischemia, whereas the p38β isoform was deactivated.[26] Moreover, in rat heart cells expressing wild-type p38α, injury was reduced by p38 MAPK inhibition, suggesting that the selective inhibition of the p38α isoform of the p38 MAPK pathway may underlie the mechanism responsible for anti-ischemic effects observed after p38 MAPK inhibition.[10] The p38β isoform appears to play a positive role in the protection against apoptotic cell death.[23]

FR167653 is a specific inhibitor for p38 MAPK; it inhibits p38α strongly but not specifically.[15] In our study, however, FR167653 significantly suppressed phosphorylation of both the α and β p38 isoforms. Some studies have reported that p38α is mainly phosphorylated under prolonged ischemia and reperfusion conditions, such as our model; however p38β has been phosphorylated in transient ischemia, including ischemic preconditioning.[23,26-28] This shows that a specific p38 MAPK inhibitor, although not specific for the p38α isoform, was effective in prevent myocardial ischemia-reperfusion injury.[29]

Myocardial ischemia-reperfusion injury is thought to result from an intense local inflammatory response involving cellular (neutrophil, endothelial cell, and monocyte) activation and elaboration of inflammatory mediators including cytokines, chemokines, and adhesion molecules. These inflammatory mediators in turn are capable of further activating multiple cell types and thus propagating an inflammatory response. Our results indicate that in murine hearts subjected to ischemia-reperfusion, MAPKs and inflammatory transcription factors become activated, resulting in elaboration of multiple inflammatory mediators and myocardial tissue destruction. NF-κB promotes transcription of many genes that encode proteins involved in inflammation.

NF-κB activation is involved in myocardial ischemia-reperfusion injury and also ischemic preconditioning.[30] However, these previous results may not be directly extrapolated to the entire pathway in myocardial ischemia-reperfusion injury, particularly between MAPKs and NF-κB, and therefore the roles of cardiac MAPKs remain to be elucidated in these clinically relevant pathophysiologic conditions. Proinflammatory mediators correlated with myocardial ischemia-reperfusion, such as inflammatory cytokines, chemokines, and some growth factors, have been shown to activate MAPK pathways.[31] In addition, TNF-α and IL-1β can be generated by p38 MAPK–dependent pathways.[32] The focus of our study was on evaluation of p38 MAPK as a possible therapeutic target to minimize myocardial ischemia-reperfusion injury.

Ischemic heart disease and acute myocardial infarction represent a major therapeutic challenge. Mammalian cells respond to ischemia with activation of numerous cell-signaling cascades that can eventually lead to irreversible damage of the myocardium and cell death. Our studies have found that ischemia-reperfusion induce distinct regulation of specific MAPK cascades, with noted differences in the intensity and time course of their activation, as well as interspecies differences.[33] Activation of multiple parallel MAPK pathways may be important in the heart's response to cellular stress.[5] In this study, increased phosphorylations of p38 MAPK, JNK, and ERK were confirmed immediately after reperfusion.

We have previously evaluated the toxicity of long-term treatment in a murine model of FR167653 (2 and 10 mg/[kg · d] intramuscularly) daily for 18 weeks by measurement of serum levels of glutamic oxaloacetic transaminase, glutamic pyruvate transaminase, creatine kinase, alkaline phosphatase, blood urea nitrogen, and creatinine along with histopathologic assessment of brain, heart, lung, liver, and kidney. There were no abnormalities in any of these parameters at the completion of the 18-week study (data not shown). Because of the potential clinical applicability suggested by our results, additional pharmacologic studies will be important.

In conclusion, we have shown in a mouse model that myocardial ischemia-reperfusion causes rapid phosphorylation and activation of MAPKs, with subsequent NF-κB activation, which results in cytokine expression and myocardial ischemia-reperfusion injury. Pretreatment with FR167653, which specifically and potently inhibits p38 MAPK, reduces NF-κB activation and inflammatory cytokine expression, decreasing myocardial tissue injury. These data show that p38 MAPK plays a central role in the molecular events that underlie myocardial ischemia-reperfusion injury. Accordingly, inhibition of p38 MAPK activity may be a therapeutic target in the prevention of inflammatory cytokine mediated tissue destruction seen with such hyperinflammatory disorders as myocardial ischemia-reperfusion.

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